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13. ABSTRACT *(Maximum 200 Words)*

The overall goal of this project is to determine the regulatory mechanisms that activate fatty acid (FA) synthetic metabolism during malignant progression of prostate cancer, and to evaluate FA synthesis as a therapeutic target. The biosynthetic enzyme, fatty acid synthase (FAS), is expressed at elevated levels in PCa cells, and is a marker of tumors with activated FA synthesis. While FAS expression is androgen responsive, it persists or is reactivated in tumors after androgen ablation, and is high in 83% of lethal tumors examined at autopsy. Experimental model systems of PCa (*in vitro* and *in vivo*) show similar patterns of FAS activation, with increased FAS during progression to androgen independence. Tumor cell changes in fatty acid metabolism are insensitive to nutritional regulation, and tumor cells produce predominantly membrane lipids. Our recent experiments have led to the observation that growth factor signals mediated by activated kinases in PCa cells and transformation models activate the fatty acid synthesis pathway, resulting in the tumor phenotype of activated FA metabolism that is insensitive to nutritional regulation. Small molecules that inhibit FAS are cytotoxic to tumors, and the mechanism of tumor cell killing is under investigation.

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INTRODUCTION: Prostate cancer represents the second leading cause of death from cancer in American men. Elevated expression of the biosynthetic enzyme, fatty acid synthase (FAS), occurs in most biologically aggressive prostate cancers, and corresponds to increased fatty acid (FA) synthetic activity. While FAS expression is androgen responsive, it persists or is reactivated in tumors after androgen ablation. This malignancy-associated cellular function represents a novel therapeutic target, since selective inhibition of FAS by anti-metabolite drugs significantly reduces cell growth and survival. These observations lead to the hypothesis that FAS expression/FA synthesis is functionally critical in prostate carcinogenesis and malignant progression, probably through support of cell growth and survival. As androgen independence emerges, FAS expression may be upregulated by alternate signaling pathways important for prostate cancer growth, including the MAP kinase and PI 3-kinase signaling cascades. The activity observed in model systems predicts that FA synthesis inhibition will be cytotoxic to prostate carcinomas that have activated this metabolic pathway.

Specific Aim 1: To determine 1] the FAS expression/activity of prostate carcinoma model systems under androgen stimulated, acutely androgen deprived, chronically androgen deprived and androgen independent conditions, 2] the relative contributions of androgen mediated versus androgen independent mechanisms for FA pathway activation under androgen deprived conditions, and the functional significance of insulin-like growth factor stimulation of fatty acid synthase.
Specific Aim 2: To 1] characterize the susceptibility of prostate cancer models to FAS inhibition and 2] optimize the use of FAS inhibitors in preclinical xenograft models for translation to clinical trials.
Specific Aim 3: To characterize the FAS expression/activity of ex-vivo surgically resected primary prostate carcinomas correlated with Gleason grade, stage, and serum levels of androgen, PSA, insulin-like growth factors I and II, and insulin-like growth factor binding protein-3.

BODY: Progress in year 2:

Specific Aim 1:

Characterization of FAS gene expression and pathway activity under androgen independent conditions:

Our recent data suggest that, in contrast to nutritional regulation of lipogenesis in liver or adipose tissue, tumor cell changes in fatty acid metabolism are driven by increases in growth factor signaling, acting in major part through the MAP kinase and PI 3-kinase signaling cascades. This result provides a likely basis for the linkage of up-regulated fatty acid metabolism with acquisition of androgen independence, and with tumor virulence, since MAP kinase and PI 3-kinase signaling contribute to both (1-7). Considered together with the information in the literature, our recent data suggest a model, that tumors coordinately regulate growth, including metabolic functions like lipogenesis, and the associated functions of membrane synthesis, through growth factor signaling altered by malignant transformation.

MAP kinase pathway inhibition reduces FAS expression and FA synthesis in tumor cell lines. MAP kinase pathway activation could provide the common signal in neoplastic cells that primarily activates tumor FA synthesis, since it is activated by various growth factor and

oncogene signals in a wide variety of tumors. To determine whether MAP kinase signals have an important role in maintaining up-regulation of FAS in a wide spectrum of tumor cells, we evaluated the effect of MEK (MAPK/extra cellular signal-regulated kinase kinase) inhibitor, U0126, on FAS expression and fatty acid synthesis in carcinoma cell lines (Figure 1). Tumor cell FAS enzyme content was reduced by 46-74% below control levels in breast, prostate and colon carcinoma cells, after 48h of MEK inhibition. Differences in cell mass due to the growth inhibitory effects of MEK inhibition were controlled for by normalization to actin. Fatty acid synthesis was similarly down-regulated by U0126. This latter effect was achieved more rapidly than the reduction in tumor cell FAS enzyme content, and probably resulted from biochemical pathway down-regulation.

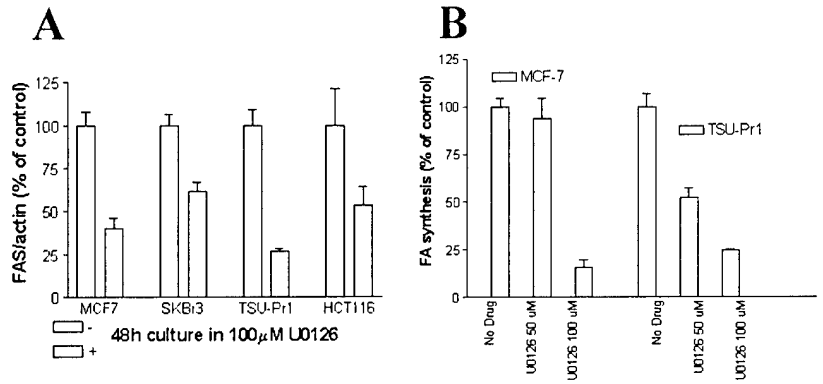

Figure 1. MAP kinase pathway signals are critical for elevated FAS expression and fatty acid synthesis in tumor cells. The MEK (MAPK/extra cellular signal-regulated kinase kinase) inhibitor, U0126, substantially reduces both FAS expression and fatty acid synthesis in carcinoma cell lines. **A.** Imunoblot analysis of FAS enzyme content in breast carcinoma cell lines, MCF7 and SKBr3, prostate carcinoma line TSU-Pr1 and colorectal carcinoma line HCT116, normalized to internal control actin immunoblot. Open bars, standard culture without drug. Gray bars, culture in 100μM U0126 for 48h. **B.** Quantitation of FA synthesis levels in MCF-7 and TSU-Pr1 carcinoma cells after overnight culture in U0126, determined by metabolic labeling with [U-^{14}C]-acetic acid followed by organic extraction of total cellular lipids. Bars: SEM.

RAS mediated transformation of human epithelial line MCF-10a produces elevated FAS expression and fatty acid synthesis. A number of investigators have studied the genetic and biochemical alterations that occur during the development of human carcinoma, using *in vitro* transformation of epithelial cells that have a normal phenotype. One approach to identifying the critical changes of neoplasia involves the transformation of human epithelial cells to carcinoma cells through the introduction of specific genes. One such gene is the H-*ras* oncogene (8-11). We adopted this *in vitro* transformation system to study transformation associated changes in fatty acid synthetic metabolism. As illustrated in Figure 2, transfection of MCF-10a cells with oncogenic H-*ras* produced foci of cells that had lost contact inhibition and had acquired transformed morphology. H-ras transformation was dependent on EGF supplementation of the culture medium, consistent with transformation resulting from augmentation in ligand-dependent signaling through the EGFR. Analysis of FAS expression in these transformed foci, and of FAS expression and fatty acid synthesis in cloned sublines demonstrated consistent up-regulation with ras transformation. This result suggested an important role for MAP kinase and PI 3-kinase pathway signaling in transformation associated up-regulation of FAS, since these signals are major downstream targets of EGFR/ras.

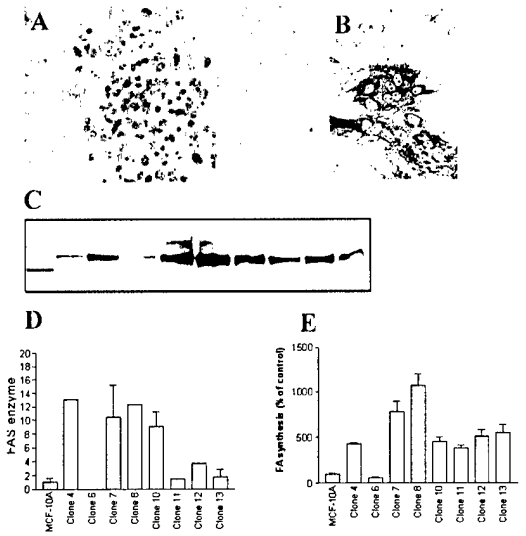

Figure 2. Expression of mutant H-*ras* induces transformation of MCF-10a cells characterized by elevated FAS expression and fatty acid synthesis. *Ras* transfected cells were plated in culture medium supplemented with 10% serum, insulin, glucocorticoids and EGF. **A.** Focus of transformed cells that show loss of contact mediated growth inhibition and nuclear atypia. **B.** Immuno-cytochemical detection of FAS enzyme in a similar focus of MCF-10a cells after *ras* transfection. **C, D, E.** A number of cloned H-*ras* transformed sublines were established. Transformed clones showed coordinate elevation of FAS enzyme content and fatty acid (FA) synthesis. **C.** Imunoblot analysis of FAS enzyme content in cloned sublines after *ras* transfection. Lane 1: 218 kD marker, Lane 2: MCF-10a, Lanes 3-10: clones 4,6,7,8,10,11,12,13. **D.** Quantitation of immunoblot in C, normalized to internal control actin immunoblot. Y values are fold elevation over MCF-10a. **E.** Quantitation of steady state FA synthesis levels in MCF-10a cells (control) and ras transformed clones, determined by metabolic labeling with [U-^{14}C]-acetic acid followed by organic extraction of total cellular lipids. Bars: SEM. Transformation of MCF-10a cells by *ras* was EGF dependent, since *ras* transfected cells that were cultured without EGF supplementation did not express a morphologically transformed phenotype (not shown).

Ras transformed MCF-10a cells have increased PI 3-kinase-Akt and MAP kinase signaling and increased SREBP-1 transcription factor levels. We selected one of these lines; clone 7, for detailed comparison with the parental line, MCF-10a. FAS enzyme level and FA synthesis are elevated 5-fold and 8-fold in clone 7 compared to MCF-10a. FAS expression is regulated primarily at the transcriptional level in the nutritional context (12, 13) and in tumor cells (14). Consistent with this, transient transfection of an FAS promoter-luciferase reporter construct containing base pairs –798 to +12 of the human FAS promoter (15) into these two cell lines demonstrated 5-fold higher transcription of the FAS promoter in clone 7 (Figure 3B). Since EGF signaling through activated ras is known to upregulate a number of signal transduction pathways including the phosphatidylinositol (PI) 3-kinase-Akt and MEK-1-ERK mitogen-activated protein (MAP) kinase pathways (16), we compared the steady state levels of phosphorylated (active) Akt-1 and p42/44 ERK1/2 proteins in MCF-10a and clone 7 by quantitative immunoblot analysis using phosphoprotein specific antibodies. Clone 7 cells demonstrated a 2 fold elevation in steady state levels of phospho-Akt-1 and a 3 fold elevation in steady state levels of phospho-p42/44 ERK1/2 proteins relative to MCF-10a cells (Figure 3A and B).

We and others have observed that sterol regulatory element binding protein 1 (SREBP-1) proteins, transcription factors that regulate the expression of lipogenic genes including FAS in liver and adipocytes (17, 18), are also involved in the regulation of FAS in tumor cells (15, 19). SREBPs are a family of membrane-bound transcription factors that activate genes involved in the synthesis of cholesterol and fatty acids and their uptake from plasma lipoproteins (18). SREBPs are synthesized as membrane-bound precursors. The amino terminal domain is a transcription factor of the basic helix-loop-helix-leucine zipper family, followed by a membrane attachment domain and a carboxy terminal regulatory domain. After synthesis in the endoplasmic reticulum, the SREBPs are escorted to the Golgi complex and cleaved, releasing the amino terminal domain to travel to the nucleus, where it binds to sterol regulatory elements (SREs) located in the promoters of target genes. These include the low density lipoprotein (LDL) receptor, cholesterol synthetic enzymes, and the lipogenic enzymes acetyl-CoA carboxylase (ACC), FAS, and stearoyl CoA desaturase (SCD). The proteolytic processing of SREBPs is under feedback control by cholesterol and by unsaturated FA (20). There are three isoforms of SREBP. Two of these, SREBP-1a and SREBP-1c, are derived from a single gene through alternate splicing, and exert greater effects on lipogenesis (21). The third isoform, SREBP-2, is derived from a separate gene, has 50% homology to the SREBP-1 proteins, and acts more strongly on the cholesterol biosynthetic pathway.

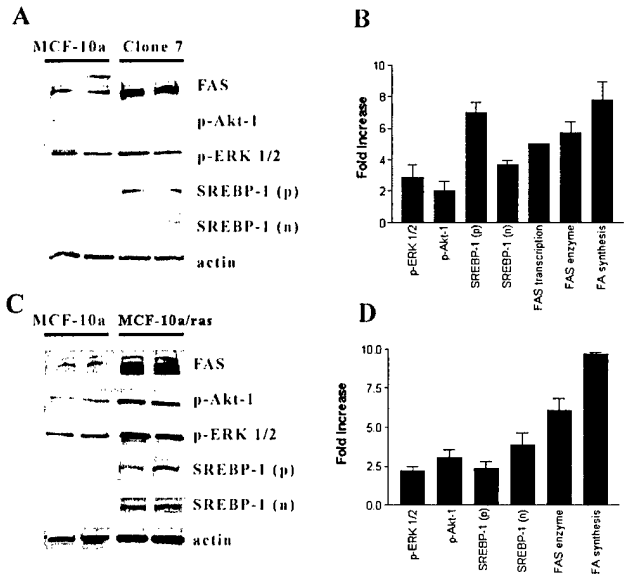

Figure 3. Ras transformed MCF-10a cells have increased PI 3-kinase-Akt and MAP kinase signaling and increased SREBP-1 transcription factor levels. A. Lysates of duplicate standard cultures of MCF-10a and clone 7 were analyzed in parallel by immunoblot for constitutive levels of FAS, of the active forms of MAP kinases p44/42 ERK 1/2 and Akt-1 (Phospho-ERK 1/2 Thr202/Tyr204, phospho-Akt-1 Ser473) and of precursor and mature forms of SREBP-1 transcription factor proteins. **B.** Quantitation of immunoblot in A; relative transcriptional activity of an FAS promoter-luciferase reporter normalized to β–galactosidase, as shown in Fig. 7B, and FA synthesis pathway activity of similar standard cultures of MCF-10a and clone 7, quantitated by metabolic labeling with [U-^{14}C]-acetic acid (23). A similar comparison of MCF-10a and a G418 selected *H-ras* transfected pool is shown in **C** and **D**.

An immunoblot analysis of MCF-10a and clone 7 demonstrated a 7 fold elevation in steady state levels of the inactive precursor form of SREBP-1, and a 3.7 fold elevation in the active, nuclear form in clone 7 cells. We determined the relative levels of FAS, SREBP-1a and SREBP-1c mRNA in clone 7 to be 5, 9 and 5 fold elevated over MCF-10a, using a quantitative real-time RT-PCR assay with normalization to GAPDH, recently developed in collaboration with Dr. P. Morin (22), not shown). A similar profile of changes to those in clone 7 were present in a pool of H-*ras* transformed MCF-10a cells generated by G418 selection after transfection (Figure 3C and D).

Akt-1 and MEK-1 can each transform MCF-10a cells and increase FAS expression. To evaluate the relative importance of PI 3-kinase-Akt and MEK-1-ERK MAP kinase pathway signaling in this transformation system, MCF-10a cells were transfected with mutant H-*ras* or with constitutively active mutants of MEK-1 (Stratagene, pFC-MEK1) or Akt-1 (generously provided by Dr. J. Testa, (24). All three oncogenes produced similar numbers of transformed foci, which demonstrated elevated FAS expression by immunocytochemistry, similar to Figure 5 (Figure 4A and not shown). Transient transfection of MCF-10a cells with any of these three oncogenes together with the FAS promoter-luciferase reporter construct containing base pairs –798 to +12 of the human FAS promoter (designated P1, Figure 4B) did not show activation of the FAS promoter over control levels. However, co-transfection with P1 and a constitutively active N-terminal truncation mutant of SREBP-1a (25) did show three-fold activation of the FAS promoter over control levels, indicating that increased levels of mature SREBP-1a are sufficient to increase expression from the FAS promoter. And deletion of the SRE/E box element at -73 to -54 (the major SREBP-1 binding site, FAS promoter mutant designated P1d20, Figure 7B) abrogated both control and SREBP-1 stimulated expression from the FAS promoter.

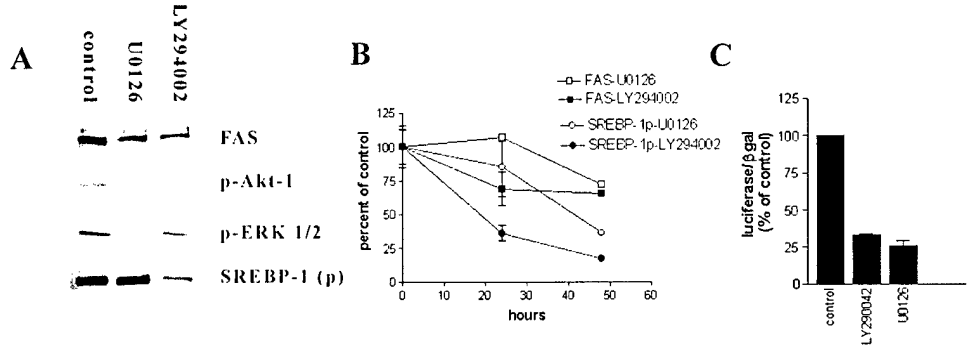

Figure 4. Inhibition of PI 3-kinase-Akt or MEK-1-ERK MAP kinase pathway signaling in H-*ras* transformed MCF-10a cells reduces FAS and SREBP-1 levels and FAS transcription. Clone 7 cells were exposed to the MEK-1 (MAPK/extra cellular signal-regulated kinase kinase) inhibitor, U0126, [100 μM], or the PI 3-kinase inhibitor, LY290042, [100 μM], for 24 or 48h, followed by imunoblot quantitation of FAS enzyme content, of the active forms of MAP kinases p44/42 ERK 1/2 and Akt-1 (Phospho-ERK 1/2 Thr202/Tyr204, phospho-Akt-1 Ser473) and of precursor and mature forms of SREBP-1 transcription factor proteins. **A.** Imunoblot analysis of FAS enzyme content at 48h, and of p-Akt-1, p-ERK 1/2, and SREBP-1 (p) at 24h. **B.** Quantitation of immunoblots from duplicate samples at 24 and 48h. Bars: SEM. **C.** Clone 7 cells were transfected with the FAS promoter-luciferase reporter construct, pGL3-P1, and CMV-βgal, and subsequently exposed to U0126 or LY290042 (both [50 μM]) for 24h, followed by measurement of luciferase and βgalactosidase activities.

Specific Aim 2:

Evaluation of FAS inhibitors, cerulenin and C-75 against *in vitro* prostate cancer models.

H-*ras* transformation sensitizes MCF-10a epithelial cells to the induction of apoptosis by FAS inhibitors, cerulenin and C-75. Finally, since earlier comparisons of unrelated cells with different levels of FA synthesis pathway activity had demonstrated increased FAS inhibitor cytotoxicity in cells with high FA synthesis pathway activity, the effects of FAS inhibitors were compared in the closely related MCF-10a and subclone 7, which differed only in the phenotype produced by H-*ras* transformation, including elevated FA synthesis. MCF-10a and subclone 7 had very similar growth rates, and both were growth inhibited by the FAS inhibitors cerulenin and C-75 (not shown). A detailed analysis of the growth inhibition using a recently developed flow cytometric assay (26) demonstrated a characteristic apoptotic response in clone 7 cells, with parallel loss of clonogenic activity (Figure 5), while MCF-10a cells had a cytostatic response as measured by flow cytometry (not shown) and retained more clonogenic activity. The pool of H-*ras* transformed MCF-10a cells generated by G418 selection after transfection shown in Figure 6 also showed increased sensitivity to FAS inhibitors in preliminary experiments (not shown).

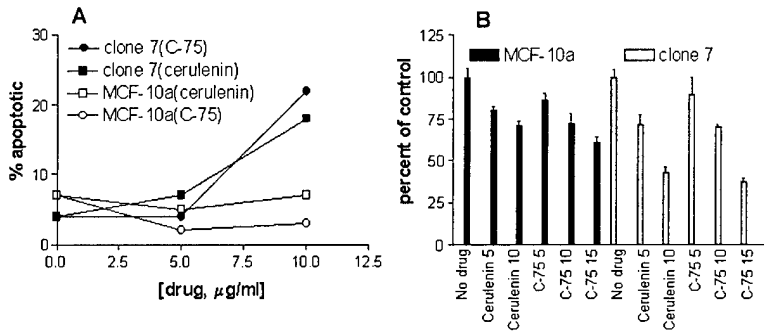

Figure 5. H-ras transformation sensitizes MCF-10a breast epithelial cells to the induction of apoptosis by FAS inhibitors, cerulenin and C-75. A. MCF-10a cells and *H-ras* transformed clone 7 were subjected to multi-parameter flow cytometry after 24 h of exposure to cerulenin or C-75 at the indicated doses. Clone 7 cells enter the apoptotic pathway in greater numbers than the parental line after inhibition of FAS. **B.** Clonogenic activity of clone 7 cells is lower than that of MCF-10a cells after 6 hour exposure to cerulenin or C-75 at the indicated doses. Determinations in triplicate, Bars:SEM.

Cytotoxic mechanisms of FAS inhibitors. Cultured cells treated with inhibitors of FAS, including the fungal product, cerulenin, and our novel compound, C-75, demonstrated a rapid reduction in FA synthesis, and a complex cellular stress response. Our current data suggest two components to this stress response: a cytotoxic effect resulting from accumulation of the committed substrate for FA synthesis, malonyl-CoA, which triggers apoptosis in tumor cells but occurs minimally in non-transformed cells, and a less potent cytostatic effect resulting from limitation of FA production. The selective toxicity of C-75 for tumor cells was sufficient to produce significant growth inhibition of human prostate cancer xenografts in immunodeficient mice (27), with only mild, reversible systemic toxicity (28).

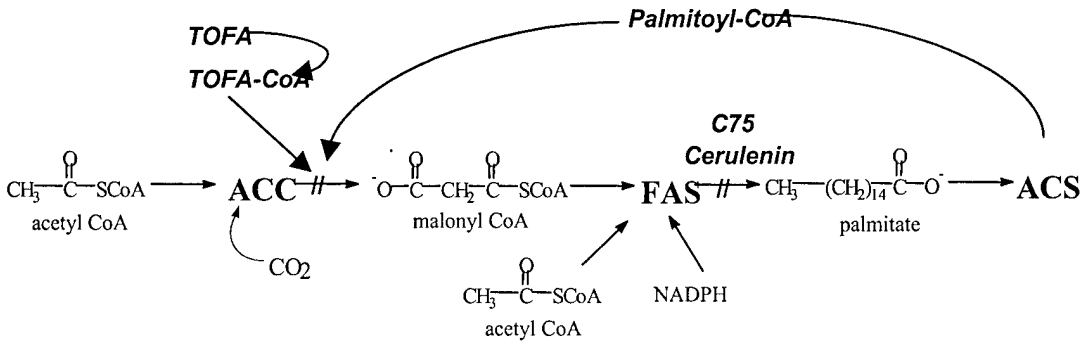

Figure 6. Inhibitors of the FA synthesis pathway. Schematic representation of the FA synthesis pathway showing the specificity of cerulenin and C-75 for fatty acid synthase (FAS) and of TOFA for acetyl-CoA carboxylase (ACC). The three FA synthesis inhibitors (2 FAS inhibitors and 1 ACC inhibitor) are all capable of reducing FA synthesis activity (incorporation of [$U^{14}C$]-acetate into extractable lipids) by comparable amounts. ACS=acyl-CoA synthase.

Malonyl-CoA is a major form of CoA in tumor cells. Our recent experiments have shown that pharmacologic inhibition of fatty acid synthesis at the physiologically regulated enzyme, acetyl-CoA carboxylase (ACC), was not cytotoxic to cancer cells. Inhibitors of the downstream enzyme, FAS, induced a rapid increase of its substrate, malonyl-CoA, while the ACC inhibitor reduced malonyl-CoA levels. Prevention of malonyl-CoA accumulation resulted in significantly reduced cytotoxicity and apoptosis, implicating malonyl-CoA accumulation in the cytotoxic mechanism. Malonyl-CoA comprises a substantial fraction of the total cellular CoA pool during normal growth of tumor cells with active FA synthesis. The several fold increased malonyl-CoA levels produced by FAS inhibition may be toxic to tumor cells by an as yet undetermined mechanism. Alternatively, malonyl-CoA accumulation may sufficiently deplete the free CoA pool in tumor cells to inhibit other critical metabolic functions that use CoA, like the Krebs cycle, mevalonate synthesis or protein acetylation. We have recently developed an assay for metabolic labeling and quantitation of total cellular CoA to facilitate further evaluation of these alternative mechanisms. Malonyl-CoA comprises about 20% of the total CoA pool in cancer cells in standard culture conditions (Figure 7).

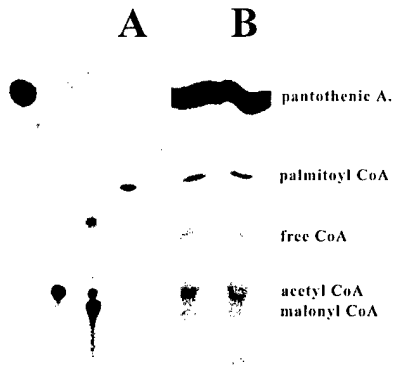

Figure 7. Constitutively elevated FA synthesis activity in cancer cells results in high steady state levels of malonyl-CoA. The total cellular CoA pool in MCF-7 carcinoma cells was labeled with ^{14}C-pantothenic acid, which is incorporated stoichiometrically into CoA, and cell extracts were separated by thin layer chromatography. **A.** Standards. **B.** Cell extracts from duplicate cultures.

Specific Aim 3:

Evaluation of FAS activity in *ex vivo* prostate cancers.

Viable tumor and benign prostate tissue was harvested from a series of radical prostatectomy specimens in an ongoing collaboration with Drs Nelson and Pflug, Dept. of Urology, University of Pittsburgh. The histologically verified tissues were metabolically labeled with ^{14}C-acetic acid followed by Folch extraction of lipids (29). Previous data had demonstrated that fatty acid synthetic activity was consistently higher in *ex vivo* primary prostate carcinoma than in benign prostate tissue, however the current data show greater variability in pathway activity (Figure 8). Efforts are currently in progress to determine whether variations in labeling protocol introduced at individual research sites, or true biological differences between clinical samples account for these results.

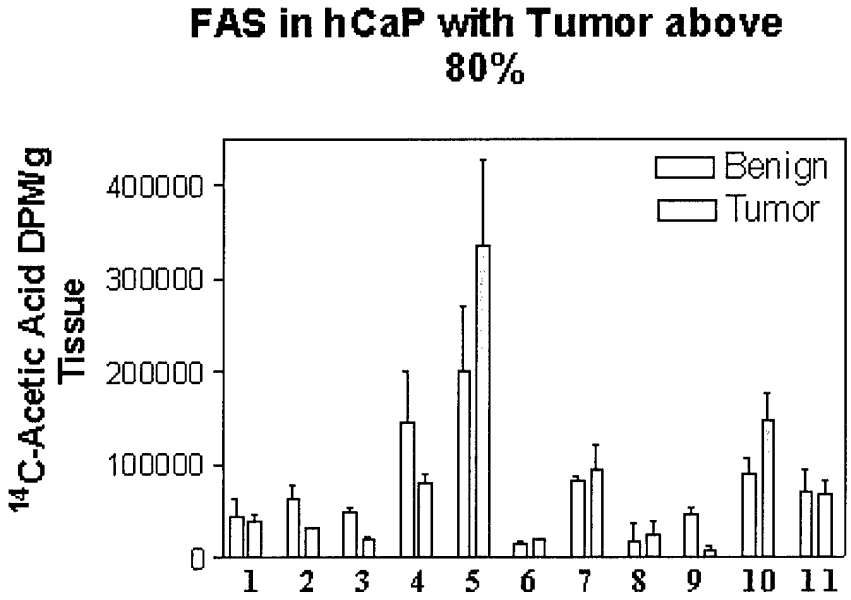

Figure 8. *Ex vivo* tissues from radical prostatectomies were transferred to labeling medium after weighing. Fatty acid synthesis was assayed with a 2 hour pulse of [U-^{14}C]-acetic acid, 1μCi/ml, followed by Folch extraction and scintillation counting. All determinations were in duplicate, except when insufficient tissue was available for replicate samples. Data are presented as mean values with bars showing the standard error.

KEY RESEARCH ACCOMPLISHMENTS, YEAR 2:

- Identification of MAP kinase and PI 3-kinase signaling as major regulators of FAS expression in advanced malignancy.
- Development of an *in vitro* model for epithelial cell transformation that recapitulates neoplastic changes in lipogenic function that are observed during PCa carcinogenesis.
- Identification of SREBP-1 transcription factors as key regulatory elements that link tumor growth signals and lipogenesis.

- Characterization of cytotoxicity of C-75 in *in vitro* systems in parallel with oncogene mediated changes in FAS activation.
- Development of biochemical assays to study the cytotoxic mechanism of FAS inhibitors.
- Metabolic labeling of primary human prostate carcinoma tissue continues.

REPORTABLE OUTCOMES, YEAR 2:

Two patent applications have been filed:

PCT/US00/31067, filed 11/20/2000,The United States Patent and Trademark Office: Increased Malonyl CoA Levels or other Manipulation of the Fatty Acid Synthesis Pathways as a Means to Selectively Kill Cancer Cells. The Johns Hopkins University School of Medicine.

PCT/US00/31068, filed 11/20/2000,The United States Patent and Trademark Office: Depletion of Cellular Coenzyme-A Levels as a Means to Selectively Kill Cancer Cells. The Johns Hopkins University School of Medicine.

CONCLUSIONS:

The data generated under this project support the following conclusions: Elevated FAS expression occurs with prostate cancer progression after androgen withdrawal. As androgen independence emerges, FAS expression may be upregulated by alternate signaling pathways important for prostate cancer growth, including the MAP kinase and PI 3-kinase signaling cascades. The SREBP-1 transcription factors are important components of this regulatory pathway. The cytotoxicity of C-75 and other FAS inhibitors increases in parallel with oncogenic changes in FAS activation. The mechanism of FAS inhibitor cytotoxicity involves fluxes in levels of intermediary metabolites derivatized to CoA . The activity observed in model systems predicts that FA synthesis inhibition will be cytotoxic to prostate carcinomas that have activated this metabolic pathway.

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